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Systemic administration of the potential countermeasure huperzine reversibly inhibits central and peripheral acetylcholinesterase activity without adverse cognitive–behavioral effects

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ABSTRACT

Huperzine A is potentially superior to pyridostigmine bromide as a pretreatment for nerve agent intoxication because it inhibits acetylcholinesterase both peripherally and centrally, unlike pyridostigmine, which acts only peripherally. Using rhesus monkeys, we evaluated the time course of acetylcholinesterase and butyrylcholinesterase inhibition following four different doses of (–)huperzine A: 5, 10, 20, and 40 µg/kg. Acetylcholinesterase inhibition peaked 30 min after intramuscular injection and varied dose dependently, ranging from about 30% to 75%. Subsequently, cognitive–behavioral functioning was also evaluated at each dose of huperzine A using a six-item serial-probe recognition task that assessed attention, motivation, and working memory. Huperzine did not impair performance, but physostigmine did. The results demonstrate that huperzine A can selectively and reversibly inhibit acetylcholinesterase without cognitive–behavioral side effects, thus warranting further study.

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1. Introduction

Current nerve agent pretreatment relies on the use of pyridostigmine (PYR) bromide tablets taken every 8 h over several days to achieve a target red blood cell (RBC) inhibition of acetylcholinesterase (AChE) of approximately 20–40% (Dunn and Sidell, 1989; Dunn et al., 1997; Ellenhorn, 1997; Kerenyi et al., 1990; Kluwe, 1987; Marino et al., 1998). PYR is a reversible carbamate AChE inhibitor that prevents some AChE from binding with the nerve agent, thereby preventing lethality. However, PYR is a polar compound that does not cross the blood–brain barrier and, thus, only inhibits peripheral AChE. Therefore, PYR does not directly protect against nerve agent-induced central nervous system (CNS) injury or centrally mediated seizures and subsequent brain damage.

Huperzine A (HUP) enters the brain and demonstrates high selectivity for AChE. These findings are based upon direct evidence from studies in which rats were decapitated at one or more times following administration of HUP and compared to saline controls using standard procedures for brain dissection followed by AChE

assays of brain homogenate by region (i.e., cortex, hippocampus, and striatum). Brain AChE inhibition was comparable and dose-dependent whether the route of administration was oral (Wang and Tang, 1998; Cheng and Tang, 1998), intraperitoneal (Tang et al., 1994), or intramuscular (Tang et al., 1989). In the case of intramuscular administration, AChE inhibition in RBC and whole brain was approximately equal and showed a high correlation up to the time of peak inhibition at approximately 30 min (Tang et al., 1989). The ability of HUP to enter the brain is also evidenced by studies that use well-documented centrally active anticholinergics to induce cognitive impairments that are then ameliorated by peripheral administration of HUP. One such controlled laboratory study utilized the anticholinergic scopolamine to induce a working-memory deficit in young rhesus monkeys then ameliorated that deficit through intramuscular administration of HUP (Ye et al., 1999). Additional evidence of the central action of HUP comes from studies in which age-related cognitive deficits were attenuated through peripheral administration of HUP in monkeys (Ye et al., 1999) and humans (for a review see Zangara, 2003). That HUP enters the brain and inhibits AChE is not in dispute (for reviews see Tang and Han, 1999; Wang, et al., 2006).

A centrally acting nerve agent pretreatment will potentially be more effective than PYR. Indeed, physostigmine (a nonpolar tertiary

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amine that penetrates the CNS) has been demonstrated to afford considerable protection against nerve agents in a variety of species (Anderson et al., 1991; Harris et al., 1991; Solana et al., 1990; von Bredow et al., 1991; Wetherell et al., 2002). More recently, several laboratories have examined HUP as a centrally acting pretreatment compound (Grunwald et al., 1994; Lallement et al., 2001, 2002a, 2002b). For example, Lallement et al. (2002b) implanted primates with an osmotic pump containing either PYR or HUP at equipotent doses to produce approximately 20% RBC AChE inhibition prior to challenge with cumulative doses of soman. Monkeys given HUP required 1.55 times more soman before the onset of convulsions and epileptic activity, demonstrating the greater efficacy of HUP against soman intoxication. HUP may be more effective than physostigmine at preventing nerve agent intoxication because it does not significantly inhibit butyrylcholinesterase (BChE), allowing this endogenous scavenger to provide protection, albeit limited, against organophosphorus nerve agents. Supporting this view, Grunwald et al. (1994) demonstrated greater protective ratios against soman challenge with huperzine relative to physostigmine.

To be used effectively as a pretreatment, a compound must be devoid of undesirable cognitive–behavioral effects. Although PYR has an excellent safety record in humans, it can produce undesirable side effects such as nausea, gastrointestinal symptoms, abdominal pain, diarrhea, excessive sweating, and frequent urination at current therapeutic levels (Dunn and Sidell, 1989). Even slight performance decrements could be significant in a battlefield scenario. The concern is even greater when the pretreatment compound acts upon the CNS. The undesirable behavioral effects of physostigmine are well documented (Bizot, 1998; Clark et al., 2005; Frederick et al., 1995; Liu, 2000; Philippens et al., 1996; Preston et al., 1985). In contrast, HUP appears to have an excellent behavioral safety profile in humans and has been evaluated for its ability to relieve memory deficits associated with Alzheimer's disease and vascular dementia (Diamond et al., 2003; Zangara, 2003). Unfortunately, the safety assessment of HUP on healthy adults (not elderly or pharmacologically challenged subjects) has been limited, and carefully controlled studies using accepted, automated, and standardized tests of cognition and performance in primates have been lacking. We endeavored to evaluate the safety of several doses of HUP on the cognitive–behavioral performance of rhesus monkeys using a computerized touchscreen task that has been shown in Department of Defense laboratories to be sensitive to cholinergic challenge. In addition, we characterized the time course of acetylcholinesterase and butyrylcholinesterase inhibition at four different doses of HUP injected intramuscularly that encompassed the therapeutically relevant dose (i.e., a dose that, like pyridostigmine, produces approximately 30% peripheral inhibition of AChE).

2. Method

The experimental protocol was approved by the Animal Care and Use Committee at the Walter Reed Army Institute of Research and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

2.1. Subjects

Six rhesus monkeys (named A1, A2, A3, A4, A5, and A7) were used to evaluate the time course of cholinesterase inhibition, two at each huperzine dose. A7 was male and weighed 7.9 kg. The remaining monkeys were female and ranged in weight from 4.4 to 5.5 kg. Only A1, A2, A3, and A4 were used to assess the behavioral effects of huperzine, and this assessment occurred several weeks after the cholinesterase time course evaluation was completed.

2.2. Drug

HUP (obtained from the Division of Biochemistry, WRAIR) was dissolved in sterile saline to a concentration of 800 µg/mL (expressed as the weight of the salt). The volume injected was varied to examine four different doses: 5, 10, 20, and 40 µg/kg. Weeks after completing the HUP assessment, physostigmine hemisulfate (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline and administered as a positive control at a dose of 70 µg/kg, expressed as the weight of the salt.

2.3. Cholinesterase evaluation

Circulating butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) were sampled from the saphenous vein of conscious monkeys restrained in a Primate Products (Immokalee, FL) restraint chair and measured using the WRAIR whole blood cholinesterase assay (Gordon et al., 2005) at the following time points: 0 (pre-injection baseline), 0.5, 1, 1.5, 2, and 24 h following intramuscular (IM) injection.

2.4. Behavioral apparatus

The subjects were tested unrestrained in their home cages (Myers and Clark, 2006). A 35.6-cm (14-in.) capacitive touch screen monitor (GoldStar StudioWorks, model GLD 451, Microtouch Systems, Inc., Methuen, MA) was attached to the front wall of each cage, with the center of the screen 38.9 cm above the chamber floor. Because screen touches are difficult to execute around the screen's perimeter, the effective area of the screen was reduced by 1.5 cm on all four sides. Banana-flavored food pellets (750 mg, Bio-Serv Inc., Frenchtown, NJ) were delivered by a pellet dispenser (BRS/LVE Model QNB-400 1) into a food cup (7.9×10.8×7.6 cm) positioned in the front of the test chamber, accessible through an aperture (7.6 cm wide×5.4 cm high) centered 15.1 cm below the lower edge of the touch screen and 11.6 cm above the chamber floor. A computer, running a custom-written Visual Basic 6.0 routine, was used to control experimental events and collect all data.

2.5. Behavioral procedure

Each daily session consisted of 240 trials and sessions lasted approximately 1 h. On each trial, six unique sample stimuli (list items) were presented sequentially, separated by a 1-s interstimulus interval (ISI) during which the screen was blank. Each list item was a compound stimulus comprised of two superimposed, randomly selected ASCII characters of different size and color. The individual characters ranged from about 0.3 to 2.7 cm in length and 0.3 to 2.7 cm in width. Because the same ASCII character could be selected for a particular sample stimulus, one character was 15% smaller than the other and was offset slightly above and to the left of the other to avoid perfect overlap and to achieve a greater diversity of compound sample stimuli. The RGB color saturation of each ASCII character ranged from 0 to 255. To exclude extremely dark characters but not true colors, at least one of the three saturation levels had to exceed 79. Each list stimulus was displayed in the top-center portion of the screen, about 13.5 cm from the left edge of the screen and about 4 cm from the top of the screen to the center of the stimulus. Each list item was presented for 3 s or until it was touched, at which point it was terminated and the ISI was initiated. After presentation of the sixth sample stimulus, the screen was blank throughout the 1-s probe delay (retention interval) that preceded the choice period. During the 15-s choice period a probe stimulus was displayed in the lower-left or lower-right portion of the screen, and a standard or default stimulus (a 6.6-cm white square) was presented in the other portion of the screen, with equal frequencies of presentation on both sides. The probe item was a compound stimulus that matched a list item on half of all trials (120). Across these “matching” trials, probe items matched

list items at each of the six serial positions with equal frequency (20 at each serial position). On matching trials, touching the probe stimulus was considered correct. In contrast, on “non-matching” trials the probe stimulus was not among those listed (novel) and touching the default stimulus was considered correct. A correct choice response immediately produced a conditioned reinforcer (the entire screen turned white for 0.25 s) every time, but produced a food pellet only 33.3% of the time, determined randomly by the computer (this probabilistic reinforcement schedule was used to maintain high, consistent levels of responding and avoid possible satiation). Touching the opposite stimulus was considered incorrect. Choice periods that elapsed without a response ended after 15 s and were considered incorrect. A 4-s intertrial interval (or ITI, during which the screen was blank) separated each trial, regardless of whether a choice was correct or incorrect. A response during the ITI reset the interval, although few such responses occurred. Only one injection was given per week to allow sufficient recovery time between doses. Sessions began exactly 30 min following injection of the test compound or saline (0.3 mL as a vehicle control). The order of doses varied across subjects in a Latin-square design.

3. Results

During the time course study, a toxic signs evaluation was conducted for each animal at each time point, and no overt clinical signs of intoxication were observed at any time.

3.1. Cholinesterase results

Fig. 1 characterizes the time course of AChE inhibition over a 24-h period for each of four doses (as differentiated in the legend). The time course of inhibition was similar across doses and approximated baseline levels by 24 h postinjection (except at the highest dose). Peak inhibition was observed at 30 min for all doses, and the peak inhibition was dose-dependent.

Fig. 2 shows peak levels of inhibition of AChE and BChE (measured at 30 min postinjection). The peak level of AChE inhibition was a function of dose and ranged from 31 to 74%. BChE inhibition ranged from 0 to 10%. This demonstrates the relative selectivity of HUP for AChE over BChE. A linear regression was conducted for BChE as a function of HUP dose, and the fit was very good. R -squared equaled .935, the slope equaled 0.333 ($p = .03$), and the y -intercept equaled -4.1 ($p = .10$, NS). For AChE, a hyperbolic model fit the data best. The formula was

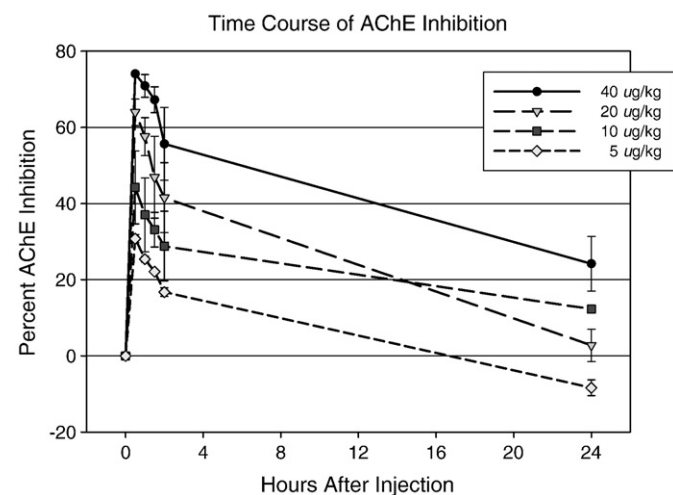


Fig. 1. Percent inhibition of AChE over 24 h following injection. Blood samples were taken at 0 (pre-injection baseline), 0.5, 1, 1.5, 2, and 24 h following IM injection of four different doses of HUP. Peak AChE inhibition occurred 30 min after injection and was dose-dependent.

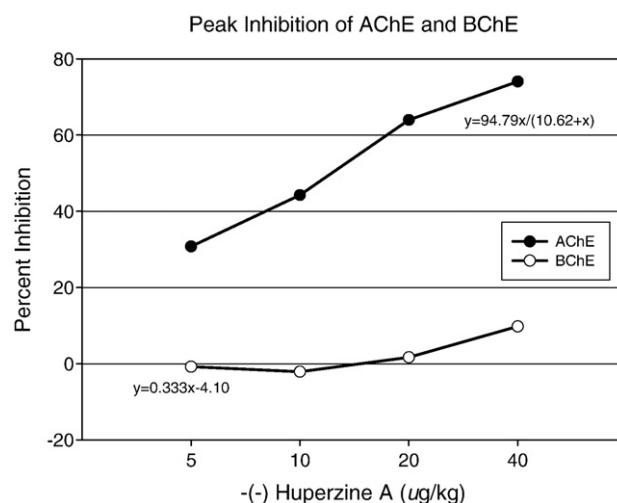


Fig. 2. Percent inhibition of AChE (filled circles) and BChE (open circles) 30 min after IM injection of HUP as a function of dose (plotted on a log scale). For doses ranging from 5 to 40 µg/kg, AChE inhibition ranged from 31 to 74% and BChE inhibition ranged from 0 to 10%.

$y = ax/(b + x)$, where a equals the asymptotic maximum and b equals the value of x producing the half-maximal response. R -squared equaled .993, a equaled 94.79 ($p = .002$), and b equaled 10.62 ($p = .015$).

3.2. Behavioral results

A repeated-measures ANOVA followed by Fisher's least significant difference procedure was used to assess significant differences ($p < .05$) as a function of drug administration. Cognitive-behavioral performance was evaluated using the serial-probe recognition task beginning 30 min after injection of each dose of HUP: 0 (saline as a vehicle control), 5, 10, 20, or 40 µg/kg. Fig. 3 shows results for each dependent measure, accuracy (top panel), trials completed (center panel), and choice reaction time (bottom panel). Compared to the saline vehicle (empty squares), cognitive-behavioral performance following HUP did not differ at any dose on any dependent measure. Thus, despite producing greater than 70% inhibition of peripheral AChE at the highest dose, HUP did not alter motivation, attention, and working memory as indexed by the serial-probe recognition task. In contrast, 70 µg/kg physostigmine did alter performance. Specifically, the number of trials completed was significantly reduced relative to all HUP doses and the saline vehicle. This reduction in trial completion was substantial, representing a greater than 60% disruption from vehicle performance. The other two performance measures, accuracy and mean choice reaction time, included only trials on which a choice response was made and were less markedly affected, only changing about 20–30% from vehicle performance. Nevertheless, physostigmine significantly impaired accuracy and choice reaction time relative to all other doses.

4. Discussion

In rhesus monkeys, we characterized the time course of peripheral AChE and BChE inhibition following four different doses of HUP that encompassed the therapeutic range of 31 to 74% AChE inhibition. The time of peak AChE inhibition equaled 30 min, regardless of dose. BChE inhibition approximated 10% at the highest HUP dose studied (40 µg/kg). Acute dosing produced no performance decrements (or improvements) in trial completion, accuracy, or choice reaction time on the SPR task. Thus, despite inhibiting AChE by as much as 74%, HUP did not produce unwanted side effects. Based on these findings, HUP appears to be behaviorally safe at therapeutic levels. In contrast, a moderate dose of physostigmine disrupted all three measures of SPR performance.

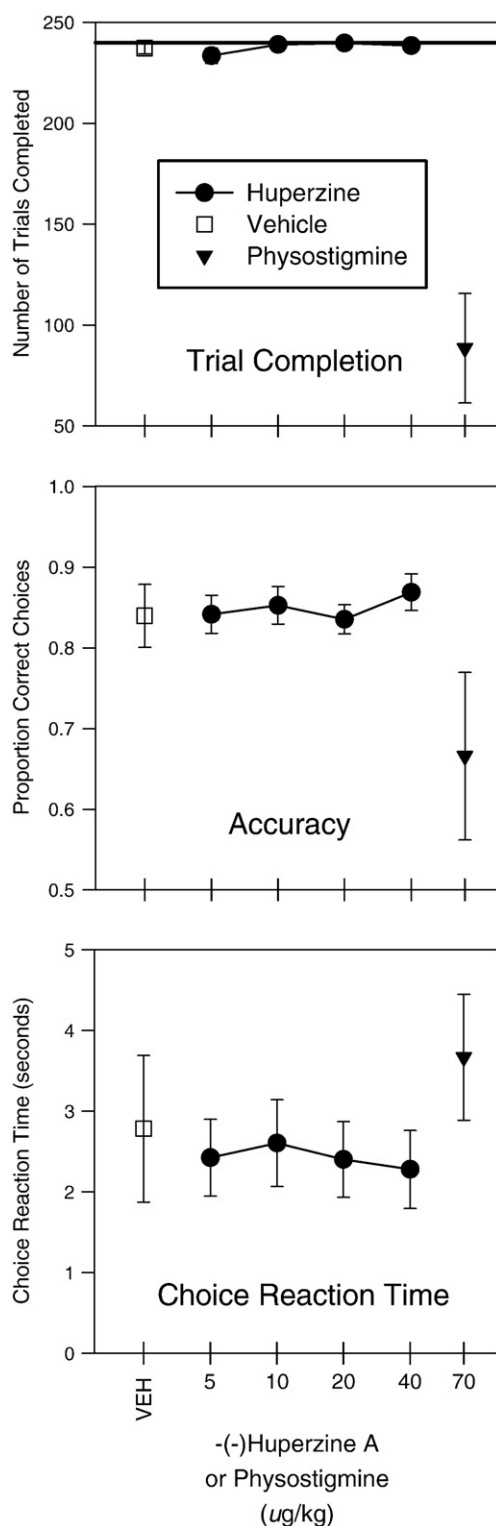


Fig. 3. Trials completed, accuracy, and choice reaction time (in seconds) as a function of dose (plotted on a log scale). Empty squares represent performance following saline injection and filled circles represent performance following the injection of HUP at the dose indicated on the x-axis.

The present results complement those of previous studies using nonhuman primates. Ye et al. (1999) administered HUP to rhesus monkeys that were either aged or pharmacologically challenged with 30 µg/kg scopolamine. Doses of 1 and 10 µg/kg HUP improved choice accuracy on a previously learned delayed spatial memory task in the elderly subjects, and doses of 10 and 100 µg/kg reversed the

scopolamine-induced deficits in the younger monkeys. Unfortunately, no data regarding cholinesterase inhibition were reported, so behavior outcomes and doses could not be correlated with AChE inhibition. However, based on AChE inhibition characterized in the present study, doses of 1 to 100 µg/kg would be expected to produce about 8–86% peak inhibition of peripheral AChE. It should be noted that generally blood AChE inhibition correlates poorly with behavior across a broad range, until a very high level of AChE inhibition is reached and behavioral impairments are reliably observed. Ye et al. also reported that the beneficial effects of HUP on choice accuracy were often observed at 20 min and 24 h (but not 48 h) after dosing, particularly at the higher doses. This suggests that the time course of inhibition may have been similar to that in the present study, with some AChE inhibition still observed at 24 h after the 40 µg/kg dose. Another key finding of the study by Ye and colleagues was that delay (retention interval) in the spatial memory task differentially modulated the drug effects on performance. Specifically, scopolamine impaired accuracy proportionally more at the longer delays, and HUP improved accuracy proportionally more at longer delays. An analogous result (differential changes in accuracy as a function of serial position) was not observed in the present study. This suggests that the manipulation of retention intervals over a range of delays (as is common in delayed matching procedures) is a useful means of detecting drug-induced changes in memory functioning. Ou et al. (2001), using similar behavioral–pharmacological procedures and young adult rhesus monkeys, extended the findings of Ye et al. to reserpine- and yohimbine-induced memory impairments, finding that 10 µg/kg HUP significantly reversed the drug-induced deficits.

It is worth noting that HUP produced no performance decrement at levels of peripheral AChE inhibition that have produced behavioral disruptions with other acetylcholinesterase inhibitors. For example, Geller et al. (1984) examined the delayed match-to-sample performance of baboons following acute soman exposure and measured peripheral AChE inhibition. Exposure to 5 µg/kg soman significantly reduced trial completion and increased response latency, and inhibited AChE by about 60–70%. Lower doses of soman (1–4 µg/kg) did not reliably disrupt performance. Chambers and Chambers (1989) exposed rats acutely to paraoxon (with atropine therapy) and produced pronounced behavioral disruptions on a fixed-ratio 10 schedule of food reinforcement. The degree of cortical AChE inhibition at these doses was about 40–60%. Philippens et al. (1992) exposed guinea pigs to acute doses of physostigmine after they acquired shuttlebox avoidance performance and measured peripheral AChE inhibition at 10, 30, and 60 min. All three doses of physostigmine significantly reduced avoidance responding, and the effect was clearly dose-dependent. For AChE inhibition, mean values ranged from 41 to 66% and the dose-dependent relation was weak. Mach et al. (2004) exposed mice acutely to a physostigmine dose producing about 50% inhibition of peripheral AChE and observed decreased locomotor activity and startle amplitude. Thus, across various species, inhibition of AChE to about 50% of pre-exposure levels can disrupt behavioral functioning. HUP may not produce behavioral deficits at comparable levels of AChE inhibition because it is more highly selective for AChE than the aforementioned cholinesterase inhibitors, thereby leaving other esterases largely unperturbed.

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The experimental protocol was approved by the Animal Care and Use Committee at the Walter Reed Army Institute of Research and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

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